A Journal of the Bangladesh Pharmacological Society (BDPS)
Journal homepage: www.banglajol.info
Abstracted/indexed in Academic Search Complete, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, Directory of Open Access Journals, EMBASE/Excerpta Medica, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index;
Bangladesh J Pharmacol 2016; 11: S106-S109
ISSN: 1991-0088

Studies on separation and properties of lumbrokinase in *Pheretima praepinguis*

Tang Mei, Liu Cao, Liang Zi, Gong Mingfu and Hu Die

College of Life Science, Leshan Normal University, Leshan, Sichuan 614 000, China.

Article Info

Received: 10 January 2016
Accepted: 8 February 2016
Available Online: 6 March 2016

DOI: 10.3329/bjp.v11iS1.26707

Cite this article:
Mei T, Cao L, Zi L, Mingfu G, Die H. Studied on separation and properties of lumbrokinase in *Pheretima praepinguis*. Bangladesh J Pharmacol. 2016; 11: S106-S109.

Abstract

The purpose of this study was to separate lumbrokinase in *Pheretima praepinguis* and examine its enzymatic properties. With *P. praepinguis* as material, lumbrokinase was separated with the salting out method. Lumbrokinase activity was measured with casein medium plate method. Effect of pH and temperature on *lumbrokinase* activity was studied. Results of lumbrokinase separated from *P. praepinguis* was relatively high. Lumbrokinase activity in neutral or slightly alkaline environment was higher. Lumbrokinase had tolerance ability to high temperature, with highly enzymatic activity under 60°C and wide range of temperature adaptation.

Introduction

Earthworm fibrinolytic enzyme, also known as lumbrokinase (Mihara et al., 1983), is a set of homoserine proteolysis enzyme with fibrinolytic activity derived from earthworm. With a strong anti-thrombotic and thrombolysis effect, lumbrokinase can effectively degrade fibrinogen thereby reducing blood viscosity (Zhu et al., 2009; Li, 2009). Applications of *l*umbrokinase in the field of heart and blood system diseases is very extensive because of good effect for the treatment of cerebral ischemic diseases (Qian et al., 2005; Yu et al., 2009), cardiovascular disease (Wei, 2010), diabetes (Hang, 2009; Chu et al., 2008) etc. Lumbrokinase may have multiple single component (Chu et al., 2008; Wang et al., 2003), the enzyme activity is affected by many factors (Wang et al., 2003; Chi et al., 1999; Zhou et al., 2011).

Pheretima praepinguis, known as large earthworms of Mount Emei, a unique earthworm Emeishan, belongs to *Pheretima, Megascolecinae, Megascolecidae*. The general body length of *P. praepinguis* is 30 cm, the longest up to 80 cm (Li, 1957).

In this experiment, crude products of lumbrokinase was extracted from *P. praepinguis* grew in Mount Emei. Plasmin activity of crude lumbrokinase was detected by using casein plate method (Wu and Zhao, 2004). To seek broader way of lumbrokinase biological preparation, the effects of pH and temperature on lumbrokinase activity was been further studied.

This paper was presented in the 3rd International Conference on Biomedicine and Pharmaceutics in Zhuhai, China, on December 11-13, 2015.

Materials and Methods

P. praepinguis

P. praepinguis was collected from the cool, moist, humus -rich soil altitude 500-700 meters from Baoguo Temple to Qingyin pavilion in Mount Emei, Sichuan Province, China.

Preparation of crude products of lumbrokinase

Fresh *P. praepinguis* was cleaned with water several times to remove all the dirty materials on the earth-

worms. The surface moisture was absorbed with the filter paper. The weight was taken and then added two volumes of phosphate buffer (pH 7.8). The tissue homogenizer was used to homogenize for 5 min at 4°C. Then the sample was kept standing overnight at 4°C. Finally the sample was centrifuged at 5,000 rpm for 10 min and discarded the pellet. The solid ammonium sulfate was added to the supernatant until saturation. The sample was again centrifuged at 4°C, 40000 rpm for 10 min. The precipitate collected was dissolved in a phosphate buffer solution, dialyzed with a dialysis bag and then desalted using a freeze dryer and freeze-dried to obtain a powdery substance of a large number of light-brown. The crude product was lumbrokinase, stored at 4°C temperature (Astrup and Mullertz, 1952).

Preliminary determination of lumbrokinase activity

The initial activity of lumbrokinase preform was determined by Casein plate method (Astrup and Mullertz, 1952). The size of diameter of the transparent circle represents casein hydrolyzing activity of lumbrokinase.

Determination of the activity of lumbrokinase by casein as substrate

Casein powder (1 g) was added to Tris-HCl buffer (volume: 100 mL, 0.05 mol/L, pH 7.8) and lysed by heating, so that the substrate concentration was 1%. It was incubated at 37°C. 2 mL of lumbrokinase treated enzyme solution was drawn, quickly added 2 mL 37°C pre-insulated substrate solution, shake well, continue to incubate for 30 min, and finally added 2 mL of 10% trichloroacetic acid solution to stop the reaction. The sample was kept at room temperature for 30 min, filtered and then the absorbance of filtrate was determined at a wavelength of 280 nm.

Determination of optimum pH of lumbrokinase activity

Taken lumbrokinase freeze-dried powder into enzyme solution, take 1 mL of enzyme solution were added 2 mL pH 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0 phosphate buffer, mix. After 4 hours at room temperature, the absorbance of filtrate was determined at a wavelength of 280 nm, retention of enzyme activity was calculated to determine the optimum pH of their lumbrokinase activity.

Determination of optimum temperature and thermal stability of lumbrokinase

2 mL lumbrokinase enzyme solution and 2 mL 1% concentration of casein solution respectively incubated for 10 min at different temperatures including 20, 30, 40, 50, 60, 70, 80, 90°C. Then the enzyme solution mixed with the casein solution at same temperature. Mixed solution was incubated for 30 min at the original temperature. Finally, 2 mL 10% trichloroacetic acid solution was added to the mixed solution to stop the reaction. The mixed solution was filtrated after standing at room temperature for 30 min. The absorbance of filtrate was determined at a wavelength of 280 nm, retention of enzyme activity was calculated, to determine the optimum temperature.

Two milliliters of enzyme solution was incubated at the optimum temperature conditions for different time including 2, 4, 6, 8, 10, 12, 16 and 24 hours. Then the enzyme solution was mixed with casein solution (1%; 2 mL). The mixed solution was incubated for 30 min at the original temperature. Finally, 2 mL 10% trichloroacetic acid solution was added to the mixed solution to stop the reaction. The mixed solution was filtrated after standing at room temperature for 30 min. The absorbance of filtrate was determined at a wavelength of 280 nm, retention of enzyme activity was calculated, to determine the holding time of lumbrokinase activity under optimum temperature.

Results

Crude extracts and active detection of lumbrokinase

Total 12 g freeze-drying crude product of lumbrokinase was obtained crude extract by ammonium sulfate salting precipitation method with 2053.1 g clean fresh *P. praepinguis*. Lumbrokinase yield was 0.5% which was relatively low.

Lumbrokinase activity was higher and the average diameter of the transparent circle was 15 mm with casein plate method (Figure 1).

Determination of optimum pH of lumbrokinase activity

Lumbrokinase activity was significantly affected by pH (Figure 2). Lumbrokinase activity was relatively stable

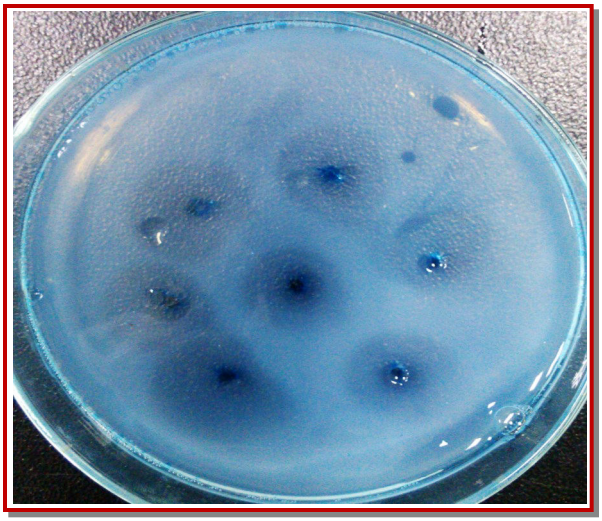

Figure 1: Lumbrokinase activity detection with casein plate method

and high activity in the alkaline environment (pH 7-9), Lumbrokinase activity retention rate maintain 75% when pH increased to 11. Lumbrokinase activity gradually reduced in an acidic environment with pH decreased, lumbrokinase activity retention rate was less than 25% at pH 4 and zero at pH 3.

Effect of temperature on lumbrokinase activity

Lumbrokinase activity was significantly affected by pH (Figure 3). Lumbrokinase activity retention rate was 100% below 60°C, rapid declined less than 10% when temperature raised 70°C and 0% when temperature reached 80°C.

Lumbrokinase activity kept intact and lumbrokinase activity retention rate maintained 100% in temperature range of 20°C to 60°C, but the water bath time of enzyme solution at 60°C also affected the lumbrokinase activity (see Figure 4). Lumbrokinase activity retention rate maintained 100% with water bath 60°C, 2 hours. With the water bath time in 60°C extended, lumbrokinase activity decreased gradually and stabilized with water bath 60°C, 16 hours. Lumbrokinase activity retention rate stabled at around 33.0% after 16 hours of water bath in 60°C.

Discussion

The yield of lumbrokinase crude extract was only 0.525%, lower than 2% in previous studies. Low lumbrokinase yield in this experiment may be related with many factors such as using fresh wet earthworms, not all discharged dirt in earthworms, earthworms homogenized stored at 4°C for long time, and other factors.

Lumbrokinase completely inactivated in pH conditions less than 3, but the stability of lumbrokinase in a neutral or alkaline environment was preferably (Yang and Ru, 1997; Lu et al., 2007). This results maybe related with protein denaturation with strong acid environment. The results in this experiment was completely consistent with the results that the clinical drug of lumbrokinase can be well adapted to alkaline environment in the

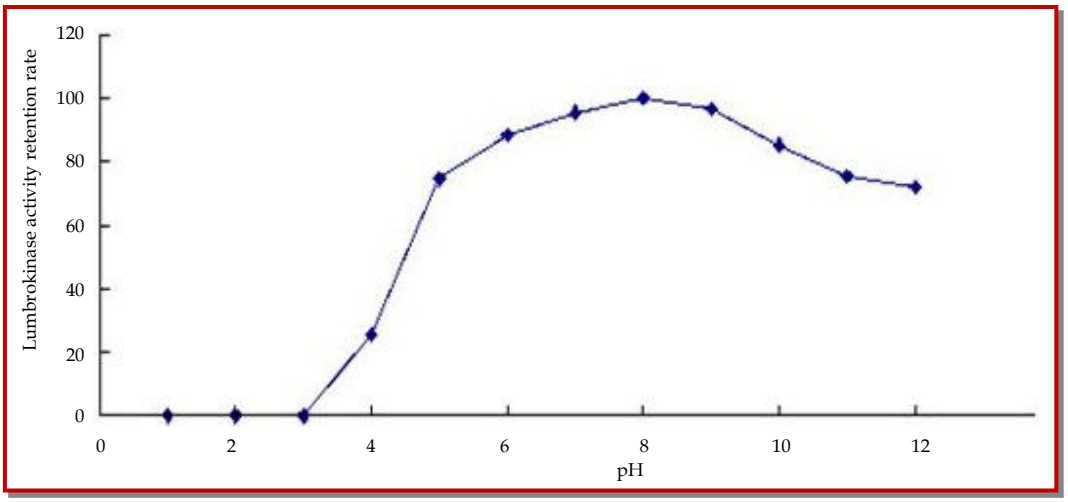

Figure 2: Effect of pH on lumbrokinase activity

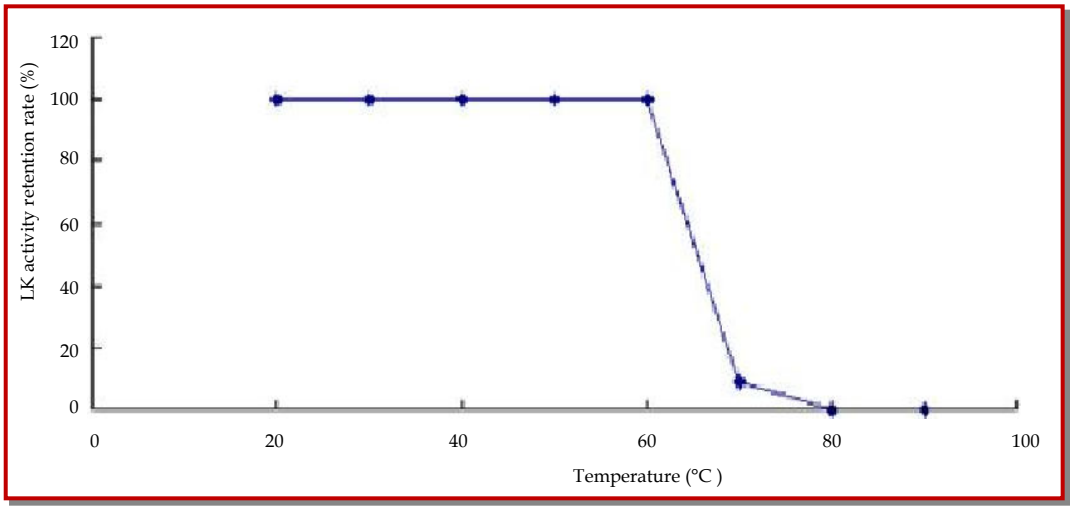

Figure 3: Effect of temperature on lumbrokinase activity

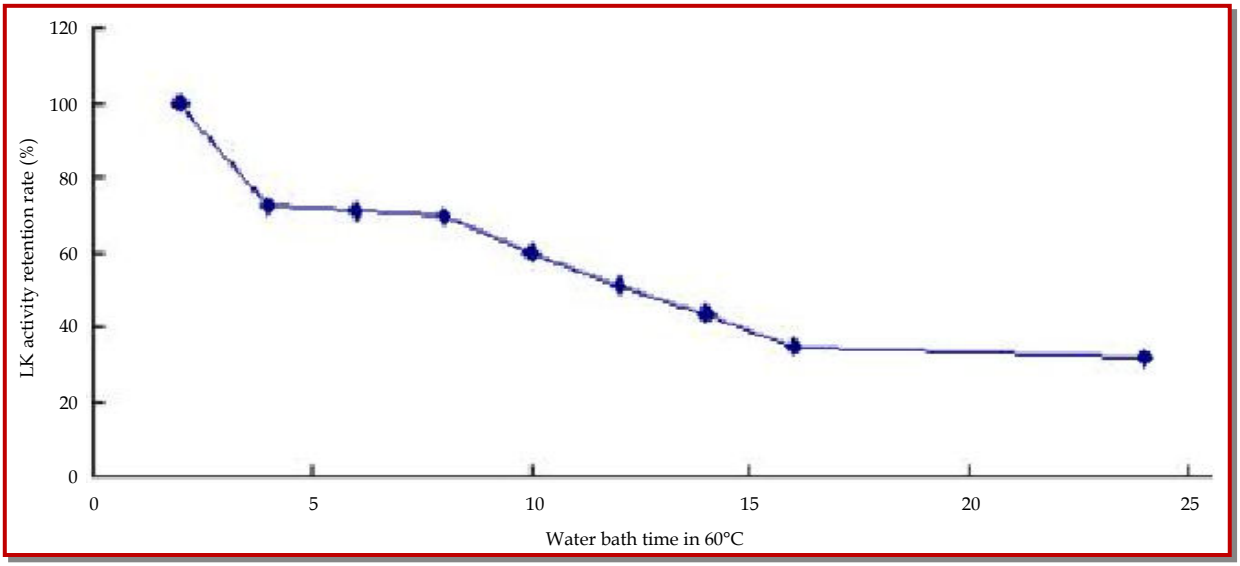

Figure 4: Effect of water bath time in 60°C on lumbrokinase activity

intestine and be inactivation in the acidic environment of gastric juice.

Lumbrokinase in this experiment has a good thermal stability, adaptation to a wide temperature range from 20°C 60°C, similar to the results described by Zhou et al (Zhou et al., 2011). Properties of lumbrokinase extracted from *P. praepinguis* and Eisenia fetida was basically same. Therefore, the process of extraction and production of the lumbrokinase can be carried out at room temperature environment directly. Oral formulations and injections made from lumbrokinase can also be directly saved at room temperature, away from heat and exposure.

Acknowledgement

This paper belongs to the project of the Key Program of Department of Education of Sichuan Province (Grant No.: 15 TD0026 and 09ZA041).

References

Chi YJ, Tian B, Zhang YH. Studies on properties and thrombolytic activity of fibrinolytic enzyme from earthworm. J Northeast Agric Univ. 1999; 30: 267-70.

Chu WP, Fu Q, Zhang MH. The effect of lumbrokinase treatment on hemorheology, glucose and lipids in type 2 diabetes mellitus. Chin J Diabetes. 2008; 16: 489-90.

Hang R. Observation of 100 treatment cases of diabetes combined microcirculation with lumbrokinase. China Prac Med. 2009; 20: 153-54.

Li YL. large earthworms in Mount Emei. Bull Biol. 1957; 5: 20-22.

Li ZY. Efficacy of ischemic stroke prevention with lumbrokinase capsules. Capital Med. 2009; 16: 58-58.

Lu L, Xu YN, Liu J, Qian M, Wang J. Clinical efficacy of injectable lumbrokinase. Acta Universitatis Medicinalis Nanjing (Nat Sci). 2007; 27: 1326-27, 37.

Mihara H, Sumi H, Akazawa T, et al., Fibrinolytic enzyme extracted from the earthworm [J]. Thromb Haemost. 1983; 50: 258-63.

Qian CM, Dang HL, Jing X. Drug analysis and treatment of lacunar brain and cerebral arteriosclerosis. J Northwest Pharmaceut. 2005; 20: 234-35.

Wang XY, Wu XB, Xu FC. Purification and some properities of fibrinolytic enzyme from *Pheretima*. Pharmaceut Biotechnol. 2003; 10: 88-91.

Wei HX. Compare treatment of angina pectoris with lumbrokinase and aspirin. Capital Med. 2010; 2: 47-48.

Wu JX, Zhao XY. Casein plate method for determination of earthworm fibrinolytic enzyme. Nat Mag. 2004; 26: 184-85.

Yang JS, Ru BG. Purification and characterization of an SDS-activated fibbrinolytic enzyme from Eisenia fetida [J]. Comp Biochen Physion. 1997; 183. 623-31.

Yu NW, Liu J, Wu JF, Xu DX, Yang YS, Guo FQ, Xie Y, Sun H. Clinical research of improve carotid atherosclerotic plaques in patients ischemic cerebrovascular disease with lumbrokinase capsule. China Med. 2009; 4: 169-70.

Zhou H, Wang CW, Zhang Y, Liu Y. Purification and characterization of lumbrokinas. Chinese J Biochem Pharmaceut. 2011; 32: 212-16.

Zhu XJ, Tan JF, Zhu Y, Ding X, Jin XY, He H, Dong P, Yang Y. Clinical studies of treatment of acute cerebral infarction with lumbrokinase capsule. Capital Med. 2009; 16: 46-47.

Author Info
Gong Mingfu (Principal contact)
e-mail: gongmingfu98@163.com

Your feedback about this paper

1. Number of times you have read this paper 0

2. Quality of paper

Excellent Good Moderate Not good

3. Your comments